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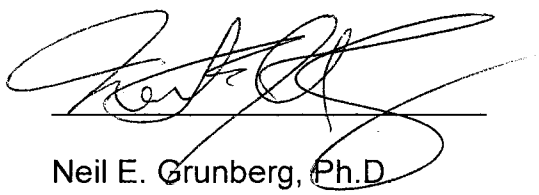

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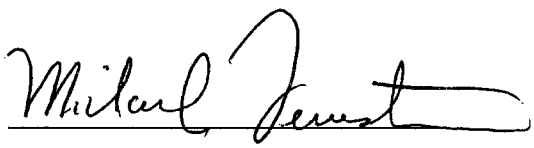

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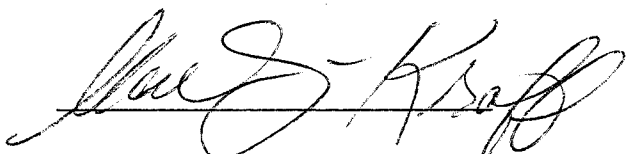

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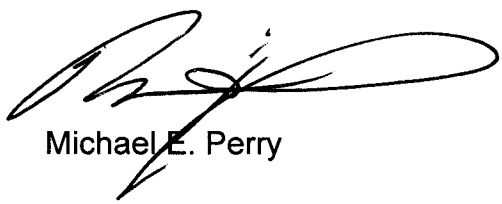

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ABSTRACT

Title of Thesis: Adolescent Rats Differ by Genetic Strain in Response to Nicotine Withdrawal

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Gender is a powerful predictor of initiation and maintenance of cigarette smoking in adults, but less is known about smoking in adolescents. This research examined nicotine withdrawal behaviors in 96 adolescent, male and female, Sprague-Dawley (SD) and Long-Evans (LE) rats. Rats received seven days continuous subcutaneous infusion of saline or nicotine hydrogen tartrate via Alzet osmotic minipumps. Behavioral observations were made before, during, and after saline or nicotine administration. SD male and female rats that received nicotine displayed significantly more withdrawal behaviors one and two days after cessation of nicotine administration compared with rats that had received saline. LE male rats that received nicotine displayed significantly more withdrawal behaviors one day (but not two days) after cessation of nicotine administration compared with males that received saline. LE females showed no significant withdrawal behaviors after cessation of nicotine administration. Results indicate that nicotine withdrawal depends on sex and genetic strain of adolescent rats.

Adolescent Rats Differ by Genetic Strain in Response to Nicotine Withdrawal

by

Michael E. Perry

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Introduction

Cigarette smoking is the leading preventable cause of death in the United States, with over 440,000 people dying every year as a result of smoking related diseases (CDC, 2004). Total annual deaths in the U.S. attributable to smoking exceed populations of entire cities such as Fort Myers, Florida; Spokane, Washington; Santa Cruz, California; and Madison, Wisconsin (US Census Bureau, 2000). Additionally, 3000 nonsmokers die of lung cancer as a result of second hand smoke every year, with an additional 300,000 youth suffering respiratory tract infections (CDC, 2004). The direct U.S. health care cost of cigarette smoking exceeds $75 billion annually, and indirect costs such as lost work productivity exceed $82 billion every year (CDC, 2005).

Despite the well-documented cost and health risks of tobacco products to smokers and those around them, over one-in-five American civilians smoke cigarettes (Trosclair, et al., 2005). In the U.S. military, nearly one third of the forces and almost 50% of the lower enlisted ranks (the military's youngest members) smoke cigarettes (Lynch et al., 2004). Age and rank are inversely correlated with cigarette smoking, with lower enlisted ranks (E1 through E3) more than nine times as likely to smoke than senior officers (O4 through O10) (Bray et al., 2002). Approximately 46% of the lower enlisted ranks — a group representing 30% of active military forces and the military's youngest members — are cigarette smokers. Most smokers are addicted to nicotine and suffer withdrawal symptoms when they abstain from tobacco use (USDHHS, 1988; USDHHS, 1994; USDHHS, 2006). Withdrawal symptoms make it difficult to

successfully maintain abstinence (USDHHS, 2000). It is important to determine whether there are sex and genetic differences in withdrawal effects of nicotine to design optimal smoking cessation strategies. The current research examined withdrawal effects of nicotine cessation in male and female adolescent rats of different genetic strains. The introduction to this paper provides the background and rationale for the present research.

The first section presents the national prevalence of health problems and costs associated with cigarette smoking and a brief overview of the impact of smoking in young populations, particularly young military members. Next, health effects of tobacco use are presented. The paper then highlights how smoking among military personnel can impact military readiness. Then, the role of cigarette smoking is reviewed, including a discussion of individual differences in tobacco use. The rationale for the present research and the value of an animal model follows with a detailed presentation of the design, hypotheses, and methods. The results of the current research are then presented. Finally, the study findings are discussed.

Health Effects of Smoking

The 2004 Surgeon General's Report on the health consequences of smoking confirms previous findings on smoking-related health risks and introduces more current information on newly discovered consequences. The findings of the report confirm that cigarette smoking results in cancers, cardiovascular diseases, respiratory diseases, and reproductive disorders (USDHHS, 2004). Cigarette smoking is implicated in additional disease states

including cataracts, hip fractures, low bone density, peptic ulcer disease, and diminished health status (USDHHS, 1990).

Over four decades of Surgeon Generals' reports indicate that smoking has been causally linked to at least ten forms of cancer including: bladder cancer (USDHHS, 1990), cervical cancer (USDHHS, 2001), esophageal cancer (USDHHS, 1982), kidney cancer (USDHHS, 1982), laryngeal cancer (USDHHS, 1980), leukemia (USDHHS, 1990), lung cancer (USDHEW, 1967), oral cancer (USDHHS, 1982), stomach cancer (USDHHS, 2004), and pancreatic cancer (USDHHS, 1990). The 1990 Surgeon General's report indicated that smoking cessation reduced cancer risk by 50 percent after only a "few years" (USDHHS, 1990, p.10).

Cigarette smoking has been causally related to many forms of cardiovascular diseases. In 1979, the Surgeon General reported that smoking was causally related to coronary heart disease (USDHEW, 1979). Subsequent reports linked smoking to other cardiovascular diseases including: abdominal aortic aneurysm (USDHHS, 1983), atherosclerosis (USDHHS, 1983), and cerebrovascular disease (USDHHS, 1989).

Respiratory diseases associated with cigarette smoking include chronic obstructive pulmonary disease (USDHEW, 1964), pneumonia (USDHHS, 1990), and respiratory effects from prenatal through adult stages of development (USDHHS, 1990). Smoking impairs lung development and function in infants, while contributing to impaired growth in children and early decline of lung function in adolescents and adults (USDHHS, 2004). There is a causal relationship

between smoking and respiratory symptoms such as coughing, wheezing, phlegm, dyspnea, and asthma (USDHHS, 2004).

Pregnant mothers who smoke harm themselves and their unborn children. Women represent over 200,000 of the Department of Defense's personnel strength of 1.3 million members (SIAD, 2006). Smoking has been linked to: fetal death and stillbirths, reduced fertility, low birth weight infants, and pregnancy complications (USDHHS, 2001). Pregnancy complications include premature membrane rupture, placenta previa, and placenta abruption (USDHHS, 2004). Additionally, there is an increased risk for preterm delivery and sudden infant death syndrome (SIDS) for mothers who smoke during pregnancy (USDHHS, 2001).

Smoking and Mental Health

An established and growing body of research reveals a relationship of mental health states such as depression and anxiety to cigarette smoking in adults and adolescents. Controversy exists in the literature, but there is evidence for a bidirectional relationship between depression and smoking (Chang, Sherritt, & Knight, 2005; Diego, Field, & Sanders, 2003; Windle & Windle, 2001). Depressed adults are up to 50% more likely to smoke cigarettes than are people who are not depressed (Vogel, Hurford, Smith, & Cole, 2003). Depressive symptoms also predict continued smoking in adolescent populations (Zhu, Sun, Choi, & Malarcher, 1999). In a study of 98 high school and college students between 16 and 18 years old, Vogel et al. (2003) suggested that adolescents might initiate smoking to gain social acceptance or to self-medicate for

depressive symptoms. The same study reported that young cigarette smokers, whose parents smoked, were likely to have higher depression scores (2003). Research involving 486 adolescents between 14 and 18 years old revealed that depression and anxiety were included in a list of seven mental health symptoms found to be more likely to exist in current cigarette smokers (Chang et al., 2005).

The research literature includes several prospective studies with evidence supporting both depression-cigarette and cigarette-depression directional relationships. Kandel and Davies (1986) reported that depressive symptoms in adolescence predict current and lifetime cigarette use. Similarly, a four-year longitudinal study by Killen et al. (1997) revealed that depression predicted later onset of smoking. Diego et al. (2003) found depression to be a significant predictor of cigarette, alcohol, and marijuana use. Conversely, Brown et al. (1996) reported that smoking predicted major depressive disorders in a sample of 1,709 adolescents. In studies involving 6,863 and 1731 adolescents, respectively, Choi et al. (1997) and Wu and Anthony (1999) also found evidence supporting a cigarette-depression relationship.

There is limited research using animal models to investigate nicotine's effects on youth. Slotkin (2002) reported that rodent exposure to nicotine during adolescence results in reduced production and function of serotonergic projections and has adverse effects on mood regulation. Further research is required to understand the extent to which cigarette smoking affects mental health or the extent to which mental health impacts cigarette smoking in youth.

Youth and Smoking

With the unprecedented wealth of information currently available to the public regarding the established health hazards of smoking and the access to this information afforded by modern technology, it is a striking fact that over one in four children smoke cigarettes (Johnston et al., 2005), with 4000 adolescents between ages 12 and 17 trying their first cigarette every day (USDHHS, 2002). One third of youth who smoke, or nearly 6.5 million, will die prematurely from health ailments related to smoking (CDC, 1996). Approximately 80% of adult cigarette smokers began smoking before they were 18 years old (USDHHS, 1994, 2000). The overall prevalence of cigarette smoking in youth is startling, but individual variables differentially affect rates of cigarette use and present a more detailed account of who might smoke.

Research indicates that smoking initiation and maintenance include biological, psychological, social, and environmental factors. Based on large-scale, self-reported data sets, Kandel and colleagues (2004) reported that gender, race, and ethnicity differentially affect smoking behavior in adolescents, suggesting possible genetic differences in the effects of nicotine, the primary addictive agent in tobacco. Difranza (2007) suggested that as many as 25% of young, first-time smokers may experience withdrawal symptoms or need to exert effort in order to quit after smoking just one cigarette. Although most smokers begin smoking during adolescence, there has been little empirical research investigating differential effects of nicotine withdrawal (a marker of addiction and a deterrent to abstinence) in adolescents, including gender and genetic

differences, and whether any such differences affect the prevalence of cigarette smoking among sub-populations of American youth. Such research is vitally important to institutions, such as the military services, whose livelihood rests on the health and readiness of its adolescents and young adults.

Military Readiness

Adolescence is broadly considered to be the period of life between the beginning of sexual maturation (puberty) and adulthood, generally between ages 13 and 19 (Marshall, 2006). In a given year, approximately 10% of active duty military members are between the ages of 17 and 19. The majority of adolescents entering military service have attained only a high school level education with fewer than 10% having any college courses (Maxfield, 2005). The relationship between smoking behavior and education level presents a problem for the military from the first day of initial training, because about 32% of young adults who do not attend college smoke a half-pack of cigarettes or more each day (Johnston, O'Malley, & Bachman, 2005). Cigarette smoking presents challenges to military readiness by leading to short-term health problems in young recruits, including shortness of breath from reduced lung function, wheezing, asthma, coughing, reduced overall physical fitness, and greater susceptibility to severe respiratory illnesses (USDHHS, 2004). Each service restricts smoking during at least the first weeks and months of initial entry training. For example, the U.S. Army bans smoking by initial entry trainees until they have completed indoctrination training and earned the rights and privileges of permanent personnel, normally after nine or more weeks of training (TRADOC,

2005). Smoking is related to early discharge in all of the military services, probably because of a constellation of factors including the tendency for smokers to have a lower family income and the association with a number of lifestyle factors such as disordered eating, drug and alcohol abuse, poor diet, and decreased physical activity (Klesges, 2001). Cigarette smoking-related attrition is estimated to be over $130 million each year (Klesges et al., 2001). For those smokers who remain in military service, the smoking-related cost to the active duty force can exceed $875 million (Klesges et al., 2001), with $8 billion in additional annual smoking-related health care costs to the Veteran's Administration (Parish, 2004). Age and rank are inversely correlated with cigarette smoking in the military, with young E1 – E3s eight times more likely to smoke than O4 through O10s (Bray et al., 2002). It is clear that the cost savings in lives and dollars might be substantial if effective methods of smoking cessation are implemented at the early stages of military training in the armed forces. Understanding the effects of withdrawal in a highly diverse force might provide insights regarding how to assist young smokers in their quit attempts and to help secure a more healthy force.

Nicotine—the Primary Addictive Agent

Nicotine dependence has been described as compulsive cigarette use to achieve pleasurable effects and to avoid withdrawal symptoms (Fagerstrom & Schneider, 1989). To help smokers abstain from tobacco use, it is critical to understand the roles of addiction and withdrawal in tobacco use. Because

nicotine is the addictive drug in tobacco (USDHHS, 1988), it is important to understand the effects of nicotine.

Cigarette smoking is the most prevalent mode of nicotine self-administration, delivering approximately 1 – 2 milligrams of nicotine per cigarette to the smoker (NIDA, 2002). When inhaled in cigarette smoke, nicotine can reach the brain in less than 10 seconds (USDHHS, 2004). Although highly addictive, nicotine is not the cause of most physical health risks of tobacco use. Burning tobacco delivers over 4,000 other chemicals, many of them highly toxic and harmful (NIDA, 2002). Among the chemicals in cigarette smoke are known carcinogens such as benzene, solvents like acetone, poisonous gases such as carbon monoxide, and polycyclic aromatic hydrocarbons (PAHs) — a group of over 100 chemicals found in burning coal, oil, gas, garbage, as well as tobacco smoke (ATSDR, 1996). The introduction of these and thousands of other chemical compounds into the body have been identified with significant health risks including cancers, cardiovascular diseases, and respiratory diseases, yet addiction to nicotine drives millions to smoke every year.

Mechanisms of Addiction

DiFranza et al. (2000) offer an explanation of nicotine dependence based on neurological pathways, suggesting that nicotine causes an increase in cholinergic receptors in the brain structures associated with the reward pathway. This up-regulation of receptors increases after the second dose of nicotine and withdrawal is noticed after the drug has been discontinued. The time frame for up-regulation of nicotinic cholinergic receptors (nAChR) in rat models has been

estimated at ten days (Schwartz et al., 1985), whereas the time required for up-regulation in human subjects, as well as up-regulation as a mechanism of dependence in humans is not clear. Further support for nAChR up-regulation was published in a study in which adolescent rats were administered nicotine by both osmotic minipump infusion to chronically administer nicotine in one group, and twice-daily nicotine injections in another group to model fluctuating plasma levels of nicotine in smokers (Abreu-Villaca et al., 2003). In both modes of administration, adolescent animals exposed to nicotine showed nAChR up-regulation in as little as two days. The effects of nicotine on receptors were evident at a rate twice that of adult animals, even when the adults were administered nicotine concentrations at three times the plasma level of the adolescents (Abreu-Villaca et al., 2003). Interestingly, continuous exposure to nicotine was not required to produce up-regulation of receptors in adolescents, as indicated by the injections administered twice a day (Abreu-Villaca et al., 2003). Levin et al. (2003) reported that female rats initiating nicotine self-administration during adolescence consumed the drug at twice the rate as did rats initiating nicotine self-administration during adulthood. The increased self-administration of nicotine in these animals persisted into adulthood.

In a study of six focus groups of human adolescent smokers, O'Loughlin et al. (2002) suggested several social factors related to nicotine dependence in addition to the biological factors. According to this study, smoking patterns were often determined by the social orientation of the adolescents involved. Smoking among youth is considered a natural part of social exchange and interaction in

adolescent smokers, drawing them together around a common cause and adding substance to their encounters. In describing their experience of nicotine dependence, these youngsters most commonly expressed the feeling of need to smoke, driven by mental and physical cravings. They expressed feelings of emptiness, sensations, shakiness, and hunger. Participants of this study also were more likely to smoke when they felt stressed. Adolescents reported that abstaining while in the presence of people smoking was among the most difficult situations to tolerate. (O'Loughlin et al., 2002).

Nicotine as a Gateway Drug

Dramatic development and neurobiological changes in the brain during adolescence may influence vulnerability to the effects of so-called "gateway drugs" such as nicotine, alcohol, and marijuana (Kandel, 2006). Research is fairly consistent on the relationship of cigarette smoking and other substance use and abuse. Experimental smoking is thought to be a particularly strong indicator of subsequent daily smoking and eventual use and abuse of other drugs (e.g., cocaine and heroin) in early adulthood (Chang et al., 2005). A 1994 National Household Survey Data on Drug Abuse revealed that children who smoked cigarettes before the age of 15 were 80 times more likely to use illicit drugs than children who were nonsmokers (Lai et al., 2000). Other investigators have suggested that cigarette and alcohol use are predictors of marijuana use — a precursor to cocaine use (Diego et al., 2003).

In animal an animal model, Levin et al. (2003) reported that nicotine self-administration by adolescent rats had far stronger and longer lasting effects on

subsequent intake during adulthood that when the initial self-administration was during adulthood. Klein (2001) found that exposure to nicotine during adolescence increased vulnerability to consume opioids in adult male rats. One potential explanation for the increased likelihood of illicit drug use after nicotine exposure is a biological mechanism, such as the enhancement of dopaminergic and serotonergic responses (Elliott & Grunberg, 2004). Some yet unknown constituent of tobacco inhibits the enzyme monoamine oxidase, which breaks down dopamine. Because dopamine is thought to stimulate pleasure in the brain, the excess levels produced by the absence of monoamine oxidase may stimulate further drug-seeking behavior for drugs of abuse such as alcohol, cocaine, and heroin (Fowler et al., 1996).

Perceived Benefits of Cigarette Smoking

Well-established human study literature indicates that many smokers associate nicotine with its perceived weight-controlling properties (Grunberg, 1997; Grunberg, 1985; Haddock, 1998; Klesges et al., 1989). Many adolescents, particularly Caucasian females, tend to smoke in order to attenuate concerns about body weight (Grunberg, 1997), and in the military, body weight remains a constant concern to conform to published weight standards (Peterson & Helton, 2000; Bray et al., 2002). The perceived effect of nicotine on body weight is supported in the animal literature. Although some sex differences have been observed, rodent studies reveal that nicotine reduces body weight gains and reduces feeding (especially of high calorie sweet foods) in adult rats (male and female) and in male adolescent rats (Abreu-Villaca et al., 2003; Faraday, Elliott,

& Grunberg, 2001). Smoking cessation produces an approximate 3 to 4 kilogram weight gain in human beings that have ceased smoking compared to those who continue smoking (Williamson et al., 1991). Additional perceived benefits of cigarette smoking include control of stress and enhanced attention, which are considered valuable benefits particularly for military personnel engaged in highly stressful working, training, and combat conditions (Grunberg et al., 2001; Bray, 1999). The benefits realized by cigarette smokers present formidable obstacles when attempting to quit smoking.

Individual Differences

As previously stated, youth are particularly susceptible to nicotine's addictive effects even after smoking just a few times (DiFranza et al., 2000) or even after one cigarette (Difranza, 2006). It has been suggested that adolescents might be more likely to become nicotine dependent than adults (DiFranza et al., 2000), and several models exist to explain the mechanisms of addiction to nicotine and why individuals might differ in response to this substance. Pomerleau et al. (1993) suggested that vulnerability to nicotine addiction is a matter of individual sensitivity to nicotine. This explanation of nicotine dependence posits that social, environmental, and biological factors all combine to influence an individual's potential for continuing to smoke and becoming nicotine dependent. Pomerleau and colleagues' position also explains why some people may become dependent after few smoking episodes, whereas others might smoke for years and never become dependent. The results of research conducted by Horn et al. (2003) supported the individual sensitivity

hypothesis. A study of 365 adolescent participants revealed that 20% of the smokers in the study were considered low-dependent smokers. That is, they were not nicotine dependent (Horn et al., 2003). The number of cigarettes smoked by this group of individuals ranged from 5 to 30 cigarettes per day (Horn et al., 2003).

Two of the most powerful predictors of smoking are gender and ethnicity (Mermelstein, 1999). About 24% of adult men smoke cigarettes, whereas 18% of adult women smoke (CDC, 2006). In terms of ethnicity, the following data from the CDC (2006) outline trends by ethnic group: American Indians — 35%; Caucasians — 22%; African Americans — 21%; Hispanics — 16%; Asians — 13%. It is notable that American Indians, leading all ethnicities at 35% cigarette smoking prevalence, are more than twice as likely to smoke cigarettes as are Asians.

Until recently, adolescent boys have smoked at a higher rate than adolescent girls, but a recent Washington Post article showcased a new study in which girls, age 12 to 17, surpassed boys in cigarette smoking for the first time since records have been maintained — over three decades (Connolly, 2006). Harrell and colleagues (1998) observed that white children and those of low socio-economic status (SES) were more likely to be experimental smokers and started smoking earlier than did their African-American counterparts and children of high SES. Much is known about smoking trends and prevalence rates in various biological, psychological and social factors, but little is known about individual differences in withdrawal.

Rodent models of genetic differences in nicotine responses have been studied for over two decades. Using mice as subjects, Marks and Collins (1981, 1983, 1986, 1989) reported genetic differences in effects of nicotine on: cholinergic activation, open field activity, brain nicotinic receptors, and nicotine tolerance. Consistent with human research, animal models also reveal strain and sex differences in response to nicotine administration. Faraday, Elliott, and Grunberg (2001) and Faraday (2002) reported that male rats displayed more behaviors related to stress in general and more activity after nicotine cessation than did females. Faraday (2002) also reported strain differences in response to stress, with Sprague-Dawley (SD) rats showing differential responses to stress compared with Long-Evans (LE) rats on sex and strain.

Withdrawal

Nicotine in cigarette smoke is reported to have addictive properties similar to that of cocaine, heroine, and amphetamines (USDHHS, 1998). The willingness of tobacco users to accept the personal risks associated with smoking poses a daunting task for researchers and health care providers. The challenge is not only to counter an attractive multibillion dollar marketing and lobbying campaign by tobacco companies (FTC, 2005), but also to understand critical factors of nicotine initiation, maintenance, and withdrawal (one of the primary symptoms of nicotine addiction) (APA, 2000; Fagerstrom & Schneider, 1989).

For health care professionals, the most desirable course of action to stem the course of smoking-related illness and death might seem to be primary

prevention—complete avoidance of smoking initiation. Yet, despite measures such as the 1998 Master Settlement Agreement (MSA) with the tobacco industry, which awarded the states $246 billion to treat smoking-related illnesses and $1.5 billion to fund smoking prevention programs (R. J. Reynolds, 2005), over one in four youth still smoke (Johnston et al., 2005). The vast majority of adult smokers began smoking when they were teenagers (USDHHS, 1994), making adolescence a vital period for prevention and cessation efforts. In fact, it is important to determine the extent to which smoking cessation and withdrawal affect individuals of varying characteristics differently in order to tailor the appropriate treatment to the appropriate patients.

Withdrawal from tobacco use by humans includes irritability, cigarette craving, cognitive and attentional deficits, sleep problems, depression, restlessness, and increased appetite (NIDA, 2004). These symptoms can begin within hours of the last cigarette smoked (Jarvis, 2004). Therefore, interventions must consider and address these affects that can lead to relapse to smoking.

Human and animal studies have firmly established that adults and adolescents display symptoms of withdrawal after cessation of nicotine administration (Colby et al., 2000; DiFranza et al., 2000, 2007; Horn et al., 2003, Malin et al., 1988, 1992; Odell et al., 2004, 2006; O'Loughlin et al., 2002). However, the degree to which withdrawal occurs in adolescents and the circumstances under which they occur is an understudied area because research has largely focused on youth prevention and adult cessation (Backinger, Fagan, Matthews, & Grana, 2003). Practically, a study involving human adolescents

would be difficult to control, given the experimental rigor necessary to avoid experimental variance inherent with human study participants. The administration of nicotine or tobacco products to youth for research also would present ethical issues, in that addictive drugs and harmful chemicals may not be administered to human children for research purposes.

Interventions to Date

Smoking cessation treatments in adults have been extensively studied, with over 6000 studies and articles published since 1996 (Curry, 2003). In contrast, a 1997 review found fewer than only 20 published controlled trials targeted adolescent tobacco users (Sussman et al., 1999). Since this 1997 review, there has been little additional research focused on adolescents. The typical participant in the existing treatment outcome literature is, on average, 40 years old, reflecting considerable under-representation of youth in the treatment literature. Because of the ethical boundaries imposed by conducting nicotine and tobacco studies in youth as well as recruitment and parental consent problems (Diviak et al., 2004), this critical area of study has been largely overlooked.

For adults, the Food and Drug Administration (FDA) has approved treatments including nicotine replacement therapy (NRT) and buproprion, an antidepressant used as an aid to smoking cessation (Curry, 2003). Randomized control trials for youth using NRT and counseling treatments have recently begun, with one study suggesting a possible benefit of group counseling in the cessation of cigarette smoking in a high school setting (Adelman, 2001). Killen and colleagues (2004) conducted a randomized controlled trial of pharmacotherapy for adolescent

smoking cessation, revealing positive results supporting the safe use of medication in children to aid smoking cessation. There also is growing evidence that cognitive-behavioral intervention might be a promising approach to assist youth in smoking cessation (McDonald, Colwell, Backinger, Husten, & Maule, 2003). Much more research is needed to determine the optimal interventions for youth who smoke. An important area overlooked in the research reviewed is the effect that genetics and gender might have in the process of withdrawal and the ensuing effects on cessation. If genetic factors and gender play a meaningful role in cessation treatment, then effective treatment options might need to employ a more customized, tailored approach based on individual differences, rather than a standardized treatment for all individuals.

Animal Models of Withdrawal

Animal models are valuable in nicotine research because administering nicotine and tobacco products to children is unsafe, unethical, and difficult to control. Animal models provide a valuable alternative in a highly controlled environment. Rats, the first mammalian species domesticated for scientific research, have been used in studies dating prior to 1850 (Jacob, 1999). Roughly 500,000 published research articles have reported using rats since the mid 1960s for biomedical, physiological, behavioral and other models of the human condition (Jacob, 1999; Mashimo et al., 2005; Wistar Institute, 2005).

For over 25 years, the Grunberg laboratory has conducted research utilizing animal models of human psychological conditions. Grunberg (1982) pioneered the use of the osmotic minipumps to administer nicotine. This

laboratory has examined effects of nicotine administration and cessation, including effects on body weight, food consumption, activity, and attention (Grunberg et al., 1987; Winders & Grunberg, 1989; Faraday et al., 1999; Faraday & Grunberg, 2000). Recently, the laboratory has focused on nicotine withdrawal in adolescent, adult, male and female rats using a paradigm developed by Malin and colleagues (1992).

In this paradigm, osmotic minipumps are subcutaneously implanted into rats, administering a chronic dose of nicotine for seven days, after which the animals' withdrawal behaviors are observed and measured. Malin et al. (1992) observed that signs of nicotine withdrawal are similar to behaviors observed during opiate abstinence, such as teeth-chattering, chews, abdominal writhes, gasps, ptosis, wet shakes, and tremors. Phillips et al. (2004) replicated the Malin group's findings that adult rats exhibited certain withdrawal behaviors within hours after discontinuation of nicotine administration. In a similar study, O'Dell and colleagues (2004) implanted minipumps for seven days, but injected mecamylamine, a nicotine receptor antagonist, on day seven to precipitate withdrawal from nicotine. Withdrawal or abstinence symptoms in these rats include body shakes, abnormal posture, chews, teeth chattering, writhes, gasps, ptosis, tremors and eye blinks (Malin et al., 1992; O'Dell et al., 2004). These reports from three different laboratories indicate that nicotine withdrawal can be studied in rats, but these reports did not examine age, sex, or genetic strain. The current study attempts to add to the existing research literature by directly

examining nicotine withdrawal in different strains of adolescent male and female rodents.

The Present Research

In order the present research examined the effects of withdrawal from chronic nicotine administration in adolescent male and female rats from two genetic strains, Sprague-Dawley (SD) and Long-Evans (LE) Hooded rats to address unanswered questions related to adolescent nicotine use and addiction, Adolescent animals were used in this experiment to determine the effects of nicotine withdrawal in sexually immature, physiologically developing animals. SD rats were selected because the strain is the most widely used animal in nicotine laboratory research and has been used extensively in the Grunberg laboratory. The SD rat is also robust and has not been bred for any specific genetic characteristic. LE rats were selected because they have shown different responses to stress and to nicotine administration (Faraday, 2002; Faraday, Blakeman & Grunberg, 2005). The LE rat also has different phenotypic characteristics (color coat, skin, and eye pigmentation) that reflect underlying genetic differences from the SD rat. These strain differences, although not analogous to human ethnic differences, provide a model of genetics that include differences in physical coloration.

The dependent variables in the present research were: withdrawal behaviors (as defined in research by Malin and colleagues [1992]), body weight, and open-field locomotion (horizontal movement and center time). There were three specific aims of this research. Specific aim (1): To determine the extent to

which withdrawal behaviors are present in adolescent rats. Previous studies have established the presence of nicotine withdrawal in adult rats (Malin et al., 1992; O'Dell et al., 2004; Hamilton et al. 2005; Shafer et al., 2005). While there have been studies citing responses to nicotine administration in adolescents, there are few establishing the presence and severity of withdrawal. Specific aim (2): To determine if there are sex differences in the effects of nicotine withdrawal in adolescent rats. Sex differences have been observed in studies of nicotine administration and withdrawal in adult rats (Faraday et al., 1999; Perkins et al., 1999; Donny et al., 2000; Grunberg et al., 1988). It is important to determine if sex plays a role in adolescents similar to that of adults. Specific aim (3): To determine if there are strain differences in nicotine withdrawal. Robust strain differences in response to nicotine administration have been found in adult female SD and LE rats, with fewer differences between males of those strains (Faraday et al., 2005). Strain differences might highlight underlying genetic differences in the effects of cessation of nicotine administration — a vitally important factor which could help develop tailored approaches to cessation treatment.

Hypotheses

Specific Aim #1: To determine the extent to which withdrawal behaviors are present in adolescent rats.

Hypothesis 1. Adolescent rats will exhibit withdrawal behaviors consistent with the behaviors observed in adult rats, but with less severity. Previous research indicates that adolescent rats display withdrawal behavior after

cessation of nicotine. Withdrawal behaviors in rats include full-body shakes, tremors, chattering teeth, writhes, gasps, ptosis, and excessive/abnormal grooming (Malin et al., 1992). However, it has been suggested that the adverse effects of nicotine withdrawal might occur to a lesser degree in adolescent than in adult animals(Faraday, Elliott, & Grunberg, 2001; O'Dell et al., 2006).

Specific Aim #2: To determine if there are sex differences in withdrawal behaviors in adolescent rats after cessation of chronic nicotine administration.

Hypothesis 2: Male rats will exhibit more severe withdrawal than female rats. In a study of nicotine self-administration, Perkins (1999) reported that nicotine is often reinforcing in men, but not in women. Grunberg, Winders, and Wewers (1991) reported that men are more likely to use tobacco products than women with mixed data on success in cessation. Faraday, Elliott, and Grunberg (2001) reported that nicotine reduced body weight and feeding of adult males and females and of adolescent males, but not adolescent females. Faraday (2002) reported that male rats tend to display more stress response in general and more activity after nicotine cessation.

Specific Aim #3: To determine the extent to which withdrawal behaviors in adolescent rats after cessation of chronic nicotine administration differ between strains.

Hypothesis 3: Withdrawal will most affect male and female SD rats, followed by male LE rats, and then female LE rats. Faraday (2002) reported sex and strain differences in responses to stress with male and female SD rats and male LE rats displaying more vulnerability to stress than female LE rats.

Faraday, Elliott and Grunberg (2001) studied the biobehavioral response to chronic nicotine administration in adult and adolescent SD rats and found that, during administration and cessation, adolescent males were more sensitive than adolescent females to nicotine's activity-enhancing effects.

METHODS

Overview

The present research was conducted to examine nicotine withdrawal-related behaviors in two strains (Sprague-Dawley [SD] and Long-Evans [LE]) of male and female adolescent rats. The technique to measure withdrawal in the present work was based on Malin and colleagues (1992), but there were several key differences. In the Malin paradigm, animals are observed in a well-lit room in cages containing no bedding. Bright lights can be stressful for the albino rat and even damaging to the retina of the animal (Lawlor, 2002; Russell, 2002). The bright lights and empty cages in the Malin paradigm may have potentiated the stress caused by nicotine withdrawal. The paradigm used during the current series of experiments was modified from the Malin approach and based on Phillips et al., (2004). The animals were observed in a dimly lit room, in cages with wood-chip bedding in order to mimic, as closely as possible, the animals' home cage environment. The dim light was produced by focusing two hooded (desk-lamp style) 60 watt lights toward the observation room ceiling from approximately 12 inches away, to produce low, ambient lighting throughout the room. The goal of this approach was to minimize other possible stressors and to increase the probability that observed responses were the result of nicotine

withdrawal alone, rather than an interaction of nicotine withdrawal and the environment.

The current research consisted of separate experiments for each strain of rat because of logistical constraints precluding observation and surgical procedures on all of the animals during the prescribed window of time. The design in each experiment was a 2 (Male or Female) X 2 (Nicotine, Saline) X 4 (Baseline, Drug Administration, Withdrawal Day 1, Withdrawal Day 2). The experiment was run in four cohorts of 24 animals each (totaling 96 animals). The cohorts were organized by strain and sex: Cohort 1 — 24 SD males; Cohort 2 — 24 SD females; Cohort 3 — 24 LE males; Cohort 4 — 24 LE females. Each cohort was sub-divided into nicotine and saline groups. Animals in all cohorts were exposed to identical experimental procedures.

Independent Variables

There were four independent variables: drug administration—nicotine or saline; sex — male or female; strain — SD or LE; and time — pre-drug administration (baseline), drug administration, and post-drug administration (withdrawal).

Dependent Variables

The dependent variables were withdrawal-related behaviors, body weight, and open-field activity (horizontal activity and center time). The current section provides a brief description of each dependent variable, followed by detailed descriptions of the equipment and exact procedures used in this study.

Withdrawal Behaviors

Prior to the beginning of the experiment, all raters participating in the experiment were trained in a single group to identify behaviors consistent with nicotine withdrawal in rats. Specific withdrawal symptoms measured were determined based on calculations of the most severe withdrawal behaviors recorded by Malin et al. (1992) during a 15 – minute period one and two days after termination of nicotine infusion. After all behaviors were identified, the raters were paired and observed additional withdrawal behaviors in a single test animal for 15 - minute observation periods. After each 15 - minute observation training period, raters compared their ratings until interrater reliability was $\geq$ 90%. For official experimental observations, raters were positioned on opposite sides of the tables, with the animals in the center. Once raters were fully trained, two raters observed two animals simultaneously.

Behavioral observations were made in a quiet, dimly lit (white light) 9 x 20 ft room on two six foot tables, each capable of holding two cages (see Figure 1). Withdrawal observations were made by two independent raters who observed animals in standard (42 x 20.5 x 20 cm) clear polycarbonate cages. There was a single animal in each cage, and observation cages had wire lids, no food, no water, and no bedding. New cages were used for each animal observed to eliminate confounds due to urine and fecal signatures. Each observed withdrawal behavior was counted as a single occurrence and was recorded on a tally sheet (see Figure 13). The withdrawal behaviors were: full body shakes, diarrhea, teeth chattering, ptosis (abnormal drooping of eyelids and facial

muscles), abnormal grooming, and abnormal posture. Five uninterrupted seconds of continuous behaviors such as abnormal grooming counted as one occurrence. The animals were observed for a 15 - minute period during each phase, after which the total behaviors observed were tallied by the raters (see tally sheet). The mean of the raters' observations was calculated to determine the total withdrawal behaviors for each animal. Between four and eight animals were observed at once, depending on the number of raters available. The sequence in which the animals were observed was counterbalanced from the first to the second day of observation to reduce any error associated with observation order.

Body weight

Body weight was measured every other day using Sartorious electronic balances. To account for error created by animal movement, the balances were programmed to calculate the mean of ten measurements taken within a period of $\leq$ 5 seconds.

Body weight is relevant to many physical and mental health conditions and was used in the present work as an indicator of general health. Decreased body weight gain occurs in rats and humans during administration of nicotine (Grunberg, Bowen, & Morse, 1984; Grunberg, 1985; Winders & Grunberg, 1989). Because of the relatively low doses of nicotine used in these experiments, the body weight effects were expected to be minimal.

Open-Field Locomotion

Locomotion was measured using a Digiscan infrared photocell system by Omnitech Electronics. Activity measurements were obtained during animals' active or dark cycle for a period of one hour in a dark room. Animals were individually placed into a 40 X 40 X 30 cm clear Plexiglas arena and with a ventilated Plexiglas lid on top of the arena. Data were automatically gathered and transmitted to a computer via an Omnitech Model DCM-I-BBU analyzer. The software measured 21 activity variables, including total distance traveled, horizontal activity, and vertical activity. Chambers were cleaned between subjects with a 35% isopropyl alcohol solution.

Locomotion in the current work was used as a measure of general health and to help interpret withdrawal behavior. Nicotine withdrawal was expected to increase specific gross motor movements. Measuring locomotion helped differentiate increases in withdrawal - related behaviors from general movement, such as horizontal and vertical locomotion.

Rater Evaluation of Horizontal Movement, Vertical Movement, and Health

At the conclusion of each 15-minute observation period, each rater subjectively evaluated the amount of horizontal and vertical movement displayed by each animal on a five-point numerical scale (see Figure 13). Raters also evaluated the health of each animal on a five-point scale, based on general appearance, coat condition, eye color, tail condition and general movement. Lower numerical ratings reflected less movement and poor appearance, and higher numerical ratings reflected high rates of movement and more healthy

appearance. For example, a movement rating of "1" was assigned if an animal displayed no movement, whereas a rating of "5" was assigned for constant movement. A health rating of "1" would reflect dull coat, flaky or dark tale, cloudy eyes, and a generally ill appearance. A health rating of "5" would reflect a glossy coat, clear eyes, pink tale, and normal movement. Assessment of appearance and mood is a face-valid indicator of general health of rats. Abnormal/low-levels of movement or changes in eye, tail or coat color and texture might indicate disease or bacteria infection (AALAS, 2004).

Experimental Design and Determination of Sample Size

This research project examined nicotine withdrawal in adolescent male and female rats of two strains, SD and LE. The study was divided into four cohorts of identical size and study design in order to divide the necessary surgical procedures and observations into practical and manageable periods of time. Each experiment was conducted as a 2 (Nicotine, Saline) x 4 (Baseline, Drug Admin, Withdrawal 1, Withdrawal 2) mixed design with 12 subjects per cell.

The sample size of 96 animals (12 animals per cell) was established because previous experiments used 12 animals in each cell to observe responses to nicotine administration (Malin et al., 1992; O'Dell et al., 2004; Hamilton et al., 2005; Perry et al., 2005). These studies, which include published research literature and unpublished work in this laboratory, reported statistically significant withdrawal effects. A *post hoc* power analysis was calculated based on the previous research using drug condition as the

independent variable, yielding a very large effect size (1.45) and high power (0.96).

Research Design and Methods

The subjects were 24 male SD rats, 24 female SD rats, 24 male LE rats, and 24 female LE rats from Charles River Laboratories (Wilmington, Massachusetts). The animals were 21 to 28 days old upon arrival. Investigators have defined adolescence in the rat as 21 – 42 days for female rats and 21 – 55 days for male rats (Spear & Brake, 1983; Ojeda & Urbanski, 1994; Faraday, Elliott, & Grunberg, 2001). The duration of the experiment was 21 days. The animals were between 42 (female SD; male and female LE) to 49 (male SD) days old at the end of withdrawal observations and within late adolescence. Prior to arrival, the animals were housed with their mothers and shipped in groups of twelve. Immediately upon arrival, they were randomly single-housed because effects of nicotine administration and cessation can be altered by housing condition (Faraday et al., 1999; Grunberg et al., 2005a, 2005b; Myracle et al., 2005). The animals weighed between 63 and 88 grams at the beginning of the experiments.

Housing

Each animal was housed on hardwood chip bedding (Pine-Dri) with unrestricted access to food (Harlan Teklad 4% Mouse/Rat Diet 7001) and water. The housing room was maintained at 20°C and 60% relative humidity on a 12-hour reversed light/dark cycle (lights on at 0700 and off at 1900). Because the rat is a nocturnal animal, the reversed light cycle was maintained in order to

match the animals' normal high-activity period with the researchers' normal observation period, making collection of behavioral measures more practical. All animals were single-housed in standard polycarbonate rat cages (40 cm x 20 cm x 20 cm). This experimental protocol was approved by the USUHS Institutional Animal Care and Use Committee and was conducted in full compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (NIH Pub, 82-23, rev. 1985).

Procedure

This experiment was conducted in three phases: pre-drug administration (baseline), drug administration, and post-drug administration (withdrawal).

Pre-Drug Administration Phase

The pre-drug administration phase consisted of seven days pre-drug administration during which time there was no substance administered to the animals. On day one, the animals arrived at the laboratory animal facility and were placed into their cages. The rats were handled by humans for several minutes on the first three days after arrival in order to minimize stress during experimental procedures. On days two through four, the animals were handled each day for five minutes each. Handling reduces the stress associated with the repeated manipulation that is required to conduct behavioral measures (Chapillon et al., 2002; Levine, 2005; Tuli et al., 1995). All animals were then acclimated to the open-field chambers on Day 4 to minimize confounding responses to any stressful effects of exposure to a novel environment (Faraday & Grunberg, 2000).

Observers were trained to recognize withdrawal behaviors during a pilot study. Initially, every member of the laboratory simultaneously observed a rat after cessation of nicotine administration until each withdrawal behavior had been exhibited by the animal and the observers indicated understanding. Next, observers gathered in groups of two, each observing an individual animal, and tallied withdrawal behaviors until interrater reliability was $\geq$ 90%.

On day six of each experiment, each animal was observed by two independent raters in a novel home cage for 15 minutes each in low light conditions. Two observers were used to increase accuracy of information. Observing in low-light during the rat's active cycle in a cage with bedding was meant to produce an environment analogous to the rats' home cage, with nicotine withdrawal acting as the only source of altered behaviors. Bright lights produce added stress in rats and can also damage the retina or even blind albino rats (Lawlor, 2002; Russell, 2002). The raters observed and recorded spontaneous behaviors consistent with withdrawal to establish baseline activity levels.

Drug Administration

The drug administration phase was a seven - day period, beginning with the infusion of 3.16 mg/kg of nicotine bitartrate or saline via subcutaneously implanted Alzet osmotic minipumps (Model 2001). A nicotine dose of 3.16 mg/kg of body weight produces withdrawal effects in rats (Malin et al., 1992; Phillips et al. 2004; O'Dell et al., 2004). In each experiment, 12 animals were assigned to the nicotine condition, and 12 animals were assigned to the saline condition.

Animals were matched by body weight and baseline locomotor activity prior to being assigned to a drug condition in order to minimize error resulting from pre-experimental individual differences. Surgeries were conducted in a separate Laboratory Animal Management (LAM) procedure room equipped with anesthesia equipment and an operating table. The animals were anesthetized with isoflurane mixed with oxygen using a vaporizer with flowmeter. The percentage of isoflurane-oxygen mix was determined based on recommendations from (LAM) personnel. The animals were placed inside a Plexiglas induction chamber saturated with the anesthesia. After tail-pinch produced no reflexive movement (after approximately 2 minutes), the animals were removed from the induction chamber, placed on an absorbent surgical pad, and fitted with a nose cone attached to the vaporizer to prevent pain by delivering constant anesthesia through the entire surgical procedure. A 2 x 2.5 cm area on each animal's back was shaved with electric clippers. After swabbing with a Betadine antiseptic solution, a small cut—approximately 1 centimeter in length — was made through the skin between the withers (shoulder blades) of each animal with surgical scissors (see Figure 2), and the pumps were inserted beneath the skin, with the pump opening toward the animal's posterior. The incisions were closed with stainless steel 9 millimeter wound clips. Each animal was monitored until fully awake, alert, and able to ambulate. The order of the surgical procedures was counterbalanced in order to alternate nicotine and saline minipump implantation. On the morning of the seventh day, nicotine phase observations were made by the investigators observing each animal. The

nicotine phase ended with the surgical explant of the minipumps, following a similar procedure as the implant.

Withdrawal

The first phase of withdrawal began after the explant of the minipumps on the seventh day of drug administration. The animals were again anesthetized with isoflurane mixed with oxygen using a vaporizer with flowmeter. The animals were placed inside a Plexiglass induction chamber saturated with the isoflurane-oxygen mix. After tail-pinch produced no reflexive movement, the animals were removed from the induction chamber, placed on an absorbent surgical pad, and fitted with a nose cone attached to the vaporizer to prevent pain by delivering constant anesthesia through the entire surgical procedure. A 2 x 2.5 cm area on each animal's back was shaved immediately behind the implant site with electric clippers. After swabbing with a betadine solution, a small cut — approximately 6 millimeters in length — was made through the skin approximately 1 centimeter behind (toward the animal's posterior) the initial implant site with surgical scissors. The pumps were guided to the new incision and pressed out from beneath the skin. The incisions were closed with stainless steel 9 millimeter wound clips. Each animal was monitored until fully awake, alert, and able to ambulate. The withdrawal period began immediately at the conclusion of surgery, and initial withdrawal observations were scheduled for 20 hours after pump removal. In previous experiments, withdrawal behaviors have been most frequent at 20 hours after cessation (Malin et al., 1992; Phillips et al., 2004; O'Dell et al., 2004).

Open-field activity was measured immediately prior to withdrawal behavior observations, approximately 17.5 hours after pump removal. Open field activity was measured for a period of one hour: to monitor general locomotion and to determine whether withdrawal behaviors were unique changes in motor movements.

Withdrawal observations took place in a behavioral testing room under low light conditions with animals entered into a novel environment (standard polycarbonate cage with bedding) for a period of 15 minutes. As with the baseline observations, observations in low-light during the rat's active cycle were meant to produce an environment similar to the rats' home cage. Two observers each watched two animals and recorded each withdrawal behavior. At the conclusion of 15 minutes, the observation period was terminated and the total behaviors were tallied and recorded on a standard sheet for each animal. Observers also rated animals on activity and health on a five-point Likert-type scale. The procedure was repeated 24 hours later for the second day of withdrawal.

Data Analytic Strategy

Withdrawal behaviors were analyzed using analyses of variance (ANOVA) before, during, and after drug administration to compare animals administered nicotine and animals administered saline. Analyses of covariance (ANCOVA) evaluated withdrawal behaviors taking baseline behaviors into account. Body weight was analyzed using ANOVAs to compare body weight between groups. ANOVAs were used to analyze open-field activity—specifically horizontal activity

and center time—before drug administration and after drug administration. Multivariate analysis of variance (MANOVA) was used to analyze various open field behaviors, because the results of various measures of locomotion are interrelated.

Several strategies were used to minimize the probability of Type I error. First, the experiment was designed to provide adequate power ($\geq$ 0.80). Actual power in this study was 0.96. Type I error is minimized when sample size supports adequate power (Keppel, 1991). In addition, only if overall analyses revealed a significant main effect of interaction were subsequent analyses performed. This strategy reduces the number of statistical tests performed (Keppel, 1991; Cohen & Cohen, 1983). All tests were two-tailed with significance determined by $p < 0.05$.

Results

Withdrawal Behavior

Withdrawal behaviors were measured at four time-points during the experiments: before drug administration, during drug administration and on the days of focus for this experiment — one and two days after cessation of nicotine (or saline).

In SD rats (See Figure 3) a repeated measures analysis of variance (ANOVA) on withdrawal behaviors revealed a significant main effect for drug ($F[1, 44]=21.53$, $p < 0.001$) and a significant main effect for time ($F[1, 44]=37.45$, $p < 0.001$) (see tables 1 & 2). There also was a time x drug interaction ($F[1, 44]=10.68$, $p < 0.001$) (see Table 2). The effects of nicotine and saline differed

over phases of the experiment. Saline and nicotine SD rats differed in withdrawal behaviors on withdrawal day one (WD1) and withdrawal day two (WD2), with nicotine rats showing significantly more withdrawal behaviors when compared to saline rats (WD Day 1: $F[1, 46] = 23.72$, $p < 0.001$) (WD Day 2: $F[1, 46] = 20.18$, $p < 0.001$) (see Table 3). An analysis of covariance (ANCOVA), with baseline behaviors as the covariate, indicated that the effects of withdrawal after cessation of nicotine remained robust ($F[1, 47]=21.96$, $p < 0.001$) (see Table 4). Male SD rats showed more withdrawal behaviors than female SD rats prior to administration of nicotine ($t[46]=4.86$, $p < 0.001$) (see Table 5). There were no significant differences between male and female SD in withdrawal behaviors at one and two days after removal of the mini-pumps and cessation of nicotine administration. From baseline to withdrawal day 1 and withdrawal day 2, male SD rats administered nicotine displayed a 28% and 26% increase, respectively, in withdrawal behaviors (see Table 17). Female SD rats administered nicotine displayed 935% and 811% increases in withdrawal behavior from baseline to withdrawal day 1 and withdrawal day 2, respectively (see Table 17).

In LE rats (see Figure 4), a repeated-measures ANOVA revealed a significant main effect for time ($F[3, 44]=27.55$, $p < 0.001$) (see Table 6). There also was a time x sex interaction ($F[3, 44]=4.75$, $p < 0.05$) and a time x drug x sex interaction ($F[3, 44]=7.43$, $p < 0.001$), indicating that drug effects differ between saline and nicotine animals at some phase and that there is a sex difference based on time and drug (see Table 6). There was no significant sex difference at baseline or on withdrawal day two, but male and female LE rats

differed on withdrawal day one. Male LE nicotine rats showed significantly more withdrawal behaviors than saline animals on withdrawal day one ($F [1, 23]=12.66$, $p < 0.05$) (see Table 7). On withdrawal day two, there was no difference between male LE rats administered nicotine and saline. In contrast, for female LE rats, there was no significant difference between nicotine and saline animals on withdrawal day one ($F[1,23]=1.58$, $p=.221$) (see Table 7). Similar to male LE rats, female LE rats showed no difference in withdrawal behaviors between nicotine and saline rats on withdrawal day two.

In summary, SD rats that had received nicotine displayed significantly more withdrawal behaviors than rats that had received saline on one and two days after cessation of nicotine. There was no difference in the manner in which male and female SD rats show nicotine withdrawal, although females appear to have a lower baseline rate of behaviors, suggesting that they exhibit more severe withdrawal relative to male rats. Male and female LE rats displayed no difference at baseline. Male LE rats administered nicotine displayed significantly more withdrawal behaviors that saline rates on withdrawal day one, but no withdrawal on day two. Conversely, female LE rats displayed no significant withdrawal differences between nicotine and saline rats on either day one or day two, suggesting no effects of withdrawal for female LE rats.

Body weight

Body weight was recorded and combined to present a mean body weight of each drug condition on the last day of each experimental phase: before drug administration, drug administration, withdrawal at day 1, and withdrawal day 2.

In SD rats (see Figure 5), a repeated measures ANOVA for body weight revealed a significant main effect for time ($F[1, 44]=3674.23$, $p < 0.001$), a significant main effect for sex ($F[1, 44]=236.10$, $p < 0.001$) (Table 8), and a significant time x sex interaction ($F[1, 44]=77.20$, $p < 0.001$), revealing that body weight differed over the course of the experiment and by sex (Table 9). All animals gained weight as they grew older, but female SD rats administered nicotine weighed significantly less than females given saline by the end of the drug administration period ($F[1, 22]=5.80$, $p < 0.05$). The weight difference suggests that adolescent female SD rats might be more sensitive to the effects of nicotine on body weight than adolescent male SD rats. The difference between animals disappeared by the end of the withdrawal period (Table 10).

In LE rats (see Figure 6), a repeated-measures ANOVA for body weight revealed a significant main effect for time ($F[3, 44]=2959.66$, $p < 0.001$) and a time x sex interaction ($F[3, 44]=140.76$, $p < 0.001$), indicating that animals' weights changed over time and that there was a sex difference in weight change (see Table 11). Although animals gained weight through each phase of the experiment, female LE rats administered nicotine weighed significantly less than female LE rats administered saline, suggesting that adolescent female rats might be more sensitive to the effects of nicotine on body weight than male LE rats ($F[1,22]=5.63$, $p < 0.05$) (Table 12). The weight difference was not significant after cessation of nicotine administration.

In summary, all animals gained weight across all phases of the experiment, but female rats of both strains exhibited significantly less weight gain

when administered nicotine relative to animals administered saline. The significant differences in body weight gain in both SD and LE female rats when compared to males of the strains suggest that adolescent female rats are more sensitive to nicotine's weight-controlling properties. Upon cessation of nicotine administration, the animals administered nicotine weighed the same as animals administered saline.

Open Field Locomotion

Open-field activity was measured in open field chambers for 60 minutes, once before drug administration and once during the early stages of withdrawal. Locomotion in the current work was used as a measure of general health and to help interpret withdrawal behavior.

SD rats administered saline showed a general increase in horizontal movement from baseline to withdrawal (see Figure 7). In male and female SD rats, animals administered nicotine moved less than saline animals during the withdrawal phase ($F[1, 43]=7.08$, $p < 0.05$) (see Table 13). The increase in withdrawal behavior combined with a decrease in general movement in animals administered nicotine indicates that the withdrawal behaviors were a separate phenomenon associated with nicotine cessation and was not the result of increased general locomotion.

Different results were observed in the LE rats (see Figure 8). Male LE rats administered nicotine displayed significantly less horizontal movement than saline animals during the withdrawal period ($F[1\ 42]=17.756$, $p < 0.001$) (see Table 14), whereas there were no significant differences between the same

animals at baseline. Female LE rats displayed a general increase in behavior in both nicotine and saline conditions from the baseline phase to the nicotine phase ($F[1, 21]=30.24$, $p < 0.001$), but no difference in activity between drug conditions at baseline and withdrawal (see Table 15). The experimental conditions and methods were identical with both SD and LE strains, suggesting that the differences observed between strains were the result of underlying genetic differences in the effects of nicotine on behavior.

Rater Evaluation of Horizontal Movement, Vertical Movement, and Health

The raters observed no horizontal movement, vertical movement, or health differences by sex, strain, or phase before, during and after nicotine (and saline) administration.

Confirmation of Hypotheses

Hypothesis 1: The hypothesis that adolescent rats would exhibit withdrawal behaviors similar to those reported in adult rat research was **partially confirmed.**

Results: Adolescent SD rats administered nicotine showed withdrawal on both days of observation after cessation of nicotine administration. Adolescent male LE rats administered nicotine showed withdrawal on the first day of nicotine cessation, but not on the second day. The absence of withdrawal in female adolescent LE rats on either day was a new finding, with female LE animals statistically unaffected by nicotine administration on one and two days after nicotine administration. Several studies report nicotine withdrawal in adult male

rats (Wilmouth & Spear, 2006; O'Dell et al., 2004), but few published studies address the effects of nicotine withdrawal in adult female rats.

Hypothesis 2: The hypothesis that male rats would exhibit more severe withdrawal (as measured by total withdrawal behaviors observed) than female rats was **partially confirmed**.

Results: After nicotine administration, LE male adolescent rats displayed withdrawal behavior but female adolescent LE rats did not. However, female SD rats displayed greater percent increases in withdrawal behavior from their pre-nicotine administration levels on day 1 (935%) and day 2 (811%) than did SD males (day 1—28%; day 2—26%).

Hypothesis 3: The hypothesis that withdrawal would most affect male and female adolescent Sprague-Dawley rats, followed by male adolescent Long-Evans rats, and then female adolescent Long-Evans rats was **confirmed**.

Results: Male and female SD adolescent rats displayed withdrawal at similar levels of withdrawal on both days after cessation of nicotine administration. Male LE rats displayed withdrawal behavior similar to male and female SD rats on one day after nicotine administration, but not on the second day after nicotine administration. Female LE rats showed no statistically significant difference in withdrawal behavior on either observation day after cessation of nicotine.

Discussion

The degree to which adolescents display withdrawal and the circumstances under which they exhibit withdrawal is an understudied area

because previous research has largely focused on youth tobacco prevention and adult tobacco cessation (Backinger et al., 2003).

The results of the current work revealed significant differences in the ways that withdrawal affects adolescent rats of varying sex and genetic strain. It is clear that SD male and female rats showed significant effects of nicotine withdrawal when compared to SD males and females administered saline. Withdrawal effects persisted for two days after cessation of nicotine in male and female SD adolescent rats. Adolescent male SD rats showed similar effects of withdrawal behavior related to nicotine cessation as did SD females. After cessation of nicotine, there were no differences in withdrawal effects between male and female SD rats. However, the females showed a greater increase in withdrawal behaviors compared to baseline than did males. SD Males administered nicotine exhibited a 28% increase in withdrawal behavior from baseline on withdrawal day 1 and a 26% increase from baseline on withdrawal day 2. Female SD rats administered nicotine, however, exhibited a more than nine-fold increase (935%) from baseline in withdrawal behavior on withdrawal day 1 and an eight-fold increase (811%) from baseline on withdrawal day two. These results indicate that nicotine withdrawal has more profound effects on female SD rats when compared to male SD rats.

Male LE rats administered nicotine displayed nicotine withdrawal behaviors one day after cessation of the drug at a significantly higher rate than did male LE rats administered saline. Withdrawal effects in male LE rats during day one were comparable to withdrawal observed in SD rats. Unlike SD rats,

there were no significant withdrawal effects in male LE rats during day 2 of nicotine administration cessation. In contrast, female LE rats displayed no significant effects of withdrawal on one or two days after cessation of nicotine administration.

Faraday (2002) observed sex and strain differences in responses to stress with adult male and female SD rats and adult male LE rats displaying more vulnerability to stress than female LE rats. If abstinence from nicotine is a stressor in rats, then the results of Faraday's work were replicated with adolescents in the current research, with male SD, female SD, and male LE rats displaying withdrawal, but female LE rats exhibiting no withdrawal. The comparison of genetic strains suggests that that the time-course and effects of withdrawal differ not only by sex, but differ by genetic strain. The differences by genetic strain and sex observed in rats might provide valuable clues to the study of nicotine dependence in humans of different gender or genetic makeup.

Existing rodent studies reveal that nicotine reduces body weight gain and feeding in rats, most notably in adult rats and male adolescent rats (Abreu-Villaca et al., 2003; Faraday, Elliott, & Grunberg, 2001), findings that are consistent with well-established human study literature indicating the weight controlling properties of nicotine (Grunberg, 1997; Grunberg, 1985). In the current study, the effects of body weight were expected to be minimal, given the relatively low doses of nicotine involved. For example, Faraday et al. (2001) administered 12 milligrams of nicotine per kilogram of bodyweight in each animal, compared to 3.16 milligrams per kilogram of body weight in the current research. Still, even at

low doses of nicotine, there were effects for body weight. Total weight gain was measured on the last day of the baseline phase, the last day of the drug administration phase, and at one and two weeks after cessation of drug administration. The results revealed that seven days of nicotine administration attenuated body weight gain in female SD and female LE rats, but not in male SD and male LE rats. The similarity of weight gain effects in female SD and LE rats is especially interesting, given the dramatic differences in withdrawal effects, with female SD rats displaying withdrawal behavior but female LE rats displaying no withdrawal behavior. Previous research has reported similar effects of nicotine administration on attenuated body weight in adult male and female SD rats (Faraday et al., 2005) and adolescent male SD rats (Faraday et al., 2001). The present findings suggest that nicotine affects withdrawal behavior through different mechanisms than it affects body weight gain, therefore providing an indicator of nicotine's withdrawal effects not observable by body weight alone.

Locomotor activity was measured in open field chambers for 60 minutes, once during baseline and once during the early stages of withdrawal. Horizontal movement provides a measure of general activity and a comparison measure to verify that the increases in withdrawal behavior are not the result of a general increase in activity.

Limitations

One limitation of this particular research is the mode of nicotine administration. Because the animals used were administered a constant dose of nicotine, the method utilized did not truly capture the fluctuating plasma nicotine

concentration or self administration of cigarette smoking. This difference might have important implications to biological and behavioral responses to nicotine administration and cessation. It is unclear whether cholinergic receptors respond differently when drugs are administered constantly than when there are repeated, finite periods of administration, such as those consistent with cigarette smoking. Further research must be conducted to clarify any differences between chronic and acute administration.

When a cigarette is smoked, there are over 4000 other chemicals delivered to the body. In the present study, we delivered only one chemical — nicotine. If there are biological, behavioral, or psychological changes elicited by any of the other chemicals in tobacco smoke, then key information might have been missed.

Conclusion

The current study reveals that genetics matter when considering the effects of nicotine on adolescent rats. It is clear that nicotine has an effect on adolescent rats but might not affect all animals in the same manner. Strain, which indicates underlying genetic differences, as well as sex, are relevant variables when considering drug effects. The present findings suggest that human research of adolescent smoking should consider genetic and sex differences. Alternative pharmacological and non-pharmacological approaches based on genetics and sex might be necessary to effectively help adolescent smokers successfully abstain.

It is clear that "one size" does not "fit all" with regard to effects of nicotine in adolescent rats. These findings suggest that prevention and cessation treatment approaches must account for individual differences and not presume that one treatment is effective for everyone. The current study provides a foundation upon which to investigate tailored smoking cessation treatments for specific individuals in order to develop more effective means of eliminating smoking in youth before they become adults. Most cigarette smokers initiate smoking prior to adulthood, making adolescence the critical period for intervening and stopping the behavior. Specific details on the most probable effects of drugs on specific genotypes will inform concerned policy makers and leaders, such as military commanders, on how to best approach smoking behavior in young people. For instance, the results of the current research have highlighted that differences in appearance which point to different genetic characteristics matter a great deal. Additionally, this research has reinforced the importance of sex and drug interactions. This work makes clear the striking differential effects of drugs that might be overlooked if individual differences are not taken into account. In order for cessation efforts to be truly effective, the additional step of evaluating genetic differences and adjusting treatment to a more individually tailored approach might result in more effective and lasting treatment results. Done effectively, tailored cessation programs might conceivably save millions of lives, save billions of dollars in health care costs, and enhance the health of a nation and the world.

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APPENDIX A (Tables)

Table 1
Sprague-Dawley ANOVA—Between Subjects (Withdrawal)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Drug	1291.688	1	1291.688	21.529	.001
Sex	671.255	1	671.255	11.188	.002
Error	2639.927	44	59.998		

Table 2
Sprague-Dawley ANOVA—Within-Subjects (Withdrawal)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
time	3057.469	1	3057.469	37.451	.001
time x Drug	871.531	1	871.531	10.675	.002
time x Sex	283.297	1	283.297	3.470	.069
Error(time)	3592.115	44	81.639		

Table 3
Sprague-Dawley ANCOVA—Between-Subjects (Withdrawal Day 1 & Day 2)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Withdrawal Day 1 (Nicotine vs. Saline)					
total_bl	19.425	1	19.425	.360	.551
Drug	1278.932	1	1278.932	23.722	.000
Error	2426.054	45	53.912		
Withdrawal Day 2 (Nicotine vs. Saline)					
total_bl	186.854	1	186.854	5.992	.018
Drug	629.265	1	629.265	20.179	.000
Error	1403.261	45	31.184		

Table 4
Sprague-Dawley ANCOVA (Withdrawal w/Baseline)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
total_bl wd	30.909	1	30.909	.557	.459
Sex	1.200	1	1.200	.022	.884
Drug	1217.493	1	1217.493	21.959	.001
Error	2384.049	43	55.443		

Table 5
Independent Samples Test (Male-Female BL Withdrawal)

	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference
Baseline Withdrawal (Male vs. Female)	4.857	46	.001	5.81250	1.19684

Note: .000 = $p < 0.0001$

Table 6
Long-Evans ANOVA—Within Subject Effects (Withdrawal)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
time	2209.005	3	736.335	27.550	.001
time x Sex	380.573	3	126.858	4.746	.004
time x Drug x Sex	595.802	3	198.601	7.431	.001
Error(time)	3527.969	44	80.181		

Table 7
Long-Evans ANOVA—Between Subjects by Sex (Withdrawal)

Sex		Sum of Squares	df	Mean Square	F	Sig.
Male	Withdrawal 1	575.260	1	575.260	12.665	.002
	Withdrawal 2	10.667	1	10.667	.174	.680
Female	Withdrawal 1	88.167	1	88.167	1.584	.221
	Withdrawal 2	135.375	1	135.375	2.202	.152
	Error	1487.958	23			

Table 8
Sprague-Dawley ANOVA—Between-Subject Effects (Body weight)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Sex	40888.547	1	40888.547	236.104	.001
Error	7619.930	44	173.180		

Table 9
Sprague-Dawley ANOVA— Within Subject Effects (Body Weight)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
time	419371.507	1	419371.507	3674.229	.001
time x Drug	96.619	1	96.619	.847	.363
time x Sex	8811.176	1	8811.176	77.197	.001
time x Drug x Sex	61.080	1	61.080	.535	.468
Error(time)	5022.100	44	114.139		

Table 10
Sprague-Dawley ANOVA—Between Subjects by Sex (Body Weight)

Sex			Sum of Squares	df	Mean Square	F	Sig.
Male	Baseline Body Weight	Between Groups	1.215	1	1.215	.060	.808
		Within Groups	443.561	22	20.162		
		Total	444.776	23			
	Mean BW NIC Phase	Between Groups	13.425	1	13.425	.137	.715
		Within Groups	2155.961	22	97.998		
		Total	2169.386	23			
	Mean BW WD1	Between Groups	3.848	1	3.848	.017	.899
		Within Groups	5100.912	22	231.860		
		Total	5104.760	23			
Female	Withdrawal Ctr Time	Between Groups	62720.150	1	62720.150	1.885	.184
		Within Groups	732050.459	22	33275.021		
		Total	794770.610	23			
	Baseline Body Weight	Between Groups	7.538	1	7.538	.489	.492
		Within Groups	339.430	22	15.429		
		Total	346.967	23			
	Mean BW NIC Phase	Between Groups	331.378	1	331.378	5.800	.025
		Within Groups	1256.858	22	57.130		
		Total	1588.236	23			
	Mean BW WD1	Between Groups	313.348	1	313.348	2.061	.165
		Within Groups	3345.309	22	152.060		
		Total	3658.657	23			
	Mean BW WD2	Between Groups	1603.935	1	1603.935	2.987	.098
		Within Groups	11813.934	22	536.997		
		Total	13417.869	23			

Table 11
Long-Evans ANOVA—Within Subject Effects (Bodyweight)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
time	1087451.973	3	362483.991	2959.657	.001
time x Drug	215.153	3	71.718	.586	.626
time x Sex	51718.441	3	17239.480	140.759	.001
time x Drug x Sex	111.080	3	37.027	.302	.824
Error(time)	16166.702	44	367.425		

Table 12
Long-Evans ANOVA—Between Subjects by Sex (Bodyweight)

Sex			Sum of Squares	df	Mean Square	F	Sig.
Male	Baseline Body Weight	Between Groups	.258	1	.258	.008	.928
		Within Groups	685.247	22	31.148		
		Total	685.505	23			
	Mean BW NIC Phase	Between Groups	55.207	1	55.207	.257	.617
		Within Groups	4729.507	22	214.978		
		Total	4784.713	23			
	Mean BW WD1	Between Groups	284.970	1	284.970	.342	.565
		Within Groups	18328.589	22	833.118		
		Total	18613.560	23			
	Mean BW WD2	Between Groups	16.335	1	16.335	.024	.879
		Within Groups	15026.883	22	683.040		
		Total	15043.218	23			
Female	Baseline Horz Act	Between Groups	2196374.269	1	2196374.269	.211	.651
		Within Groups	218696639.644	21	10414125.697		
		Total	220893013.913	22			
	Baseline Body Weight	Between Groups	17.596	1	17.596	.908	.351
		Within Groups	426.443	22	19.384		
		Total	444.039	23			
	Mean BW NIC Phase	Between Groups	241.427	1	241.427	5.634	.027
		Within Groups	942.726	22	42.851		
		Total	1184.153	23			
	Mean BW WD1	Between Groups	135.057	1	135.057	1.324	.262
		Within Groups	2243.745	22	101.988		
		Total	2378.802	23			
	Mean BW WD2	Between Groups	130.387	1	130.387	.468	.501
		Within Groups	6123.472	22	278.340		
		Total	6253.859	23			

Table 13
Sprague Dawley MANOVA—Between-Subjects Effects (Horizontal Activity)

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Drug	Baseline Horz Act	4931309.798	1	4931309.798	.499	.484
	Withdrawal Horz Act	132166440.014	1	132166440.014	7.080	.011
Error	Baseline Horz Act	424523131.583	43	9872630.967		
	Withdrawal Horz Act	802661376.386	43	18666543.637		

Table 14
Long-Evans ANOVA—Between-Subjects Effects (Horizontal Activity)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
BL_Horz	2931804.146	1	2931804.146	.219	.642
Drug	16729460.525	1	16729460.525	1.252	.270
Sex	237259078.140	1	237259078.140	17.756	.001
Drug x Sex	20033000.053	1	20033000.053	1.499	.228
Error	561197612.370	42	13361847.914		

Table 15
Long-Evans ANOVA (female)—Within Subjects Effects (Horizontal Activity)

Sex	Source	Type III Sum of Squares	df	Mean Square	F	Sig.
female	phase	487217193.680	1	487217193.680	30.244	.001
	Error(phase)	338298419.277	21	16109448.537		

Table 16
Sprague-Dawley MANOVA—Between-Subjects Effects (Center Time)

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Sex	Baseline Ctr Time	152337.476	1	152337.476	9.744	.003
	Withdrawal Ctr Time	3747.871	1	3747.871	.140	.710
Drug	Baseline Ctr Time	11170.991	1	11170.991	.715	.403
	Withdrawal Ctr Time	129148.220	1	129148.220	4.811	.034
Error	Baseline Ctr Time	672255.621	43	15633.852		
	Withdrawal Ctr Time	1154265.795	43	26843.391		

Table 17
Mean withdrawal behavior and percent change from baseline

	Mean W/D Behaviors			% Change from baseline	
	Baseline	WD Day1	WD Day 2	WD Day 1	WD Day 2
Male SD saline	6.13	10.08	11.33	55%	18%
Male SD nicotine	10.92	19.42	19.58	28%	26%
Female SD saline	3.5	7.86	9.79	124%	180%
Female SD nicotine	1.92	19.88	17.5	935%	811%
Male LE saline	5.67	9	9.42	59%	66%
Male LE nicotine	3.5	18.79	10.75	437%	207%
Female LE saline	4.04	10.58	9.83	162%	143%
Female LE nicotine	2.75	6.75	14.58	145%	330%

APPENDIX B (Figures)

FIGURE 1

Observation Room Set-up

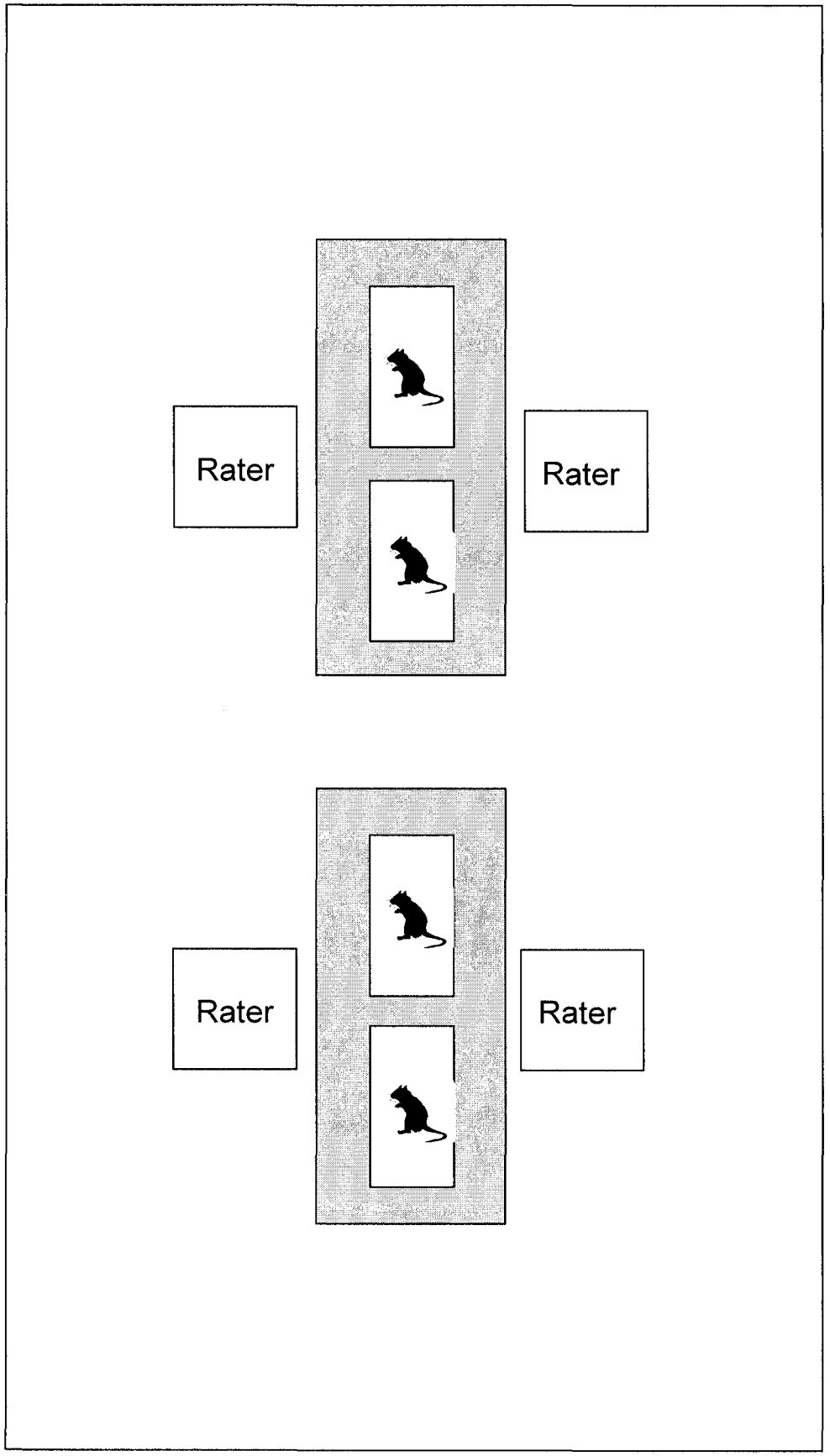

FIGURE 2

Incision and Implant Site

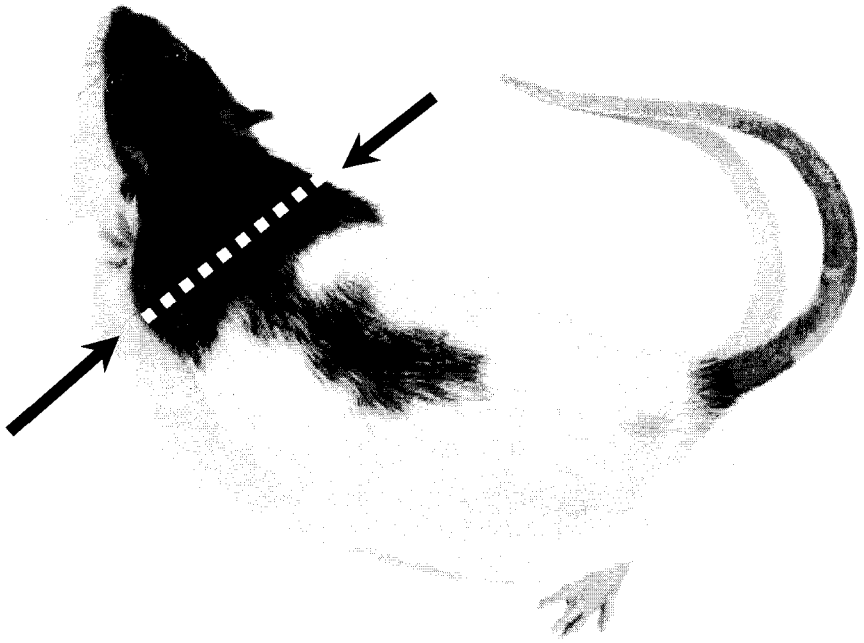

FIGURE 3

Error bars = Standard E

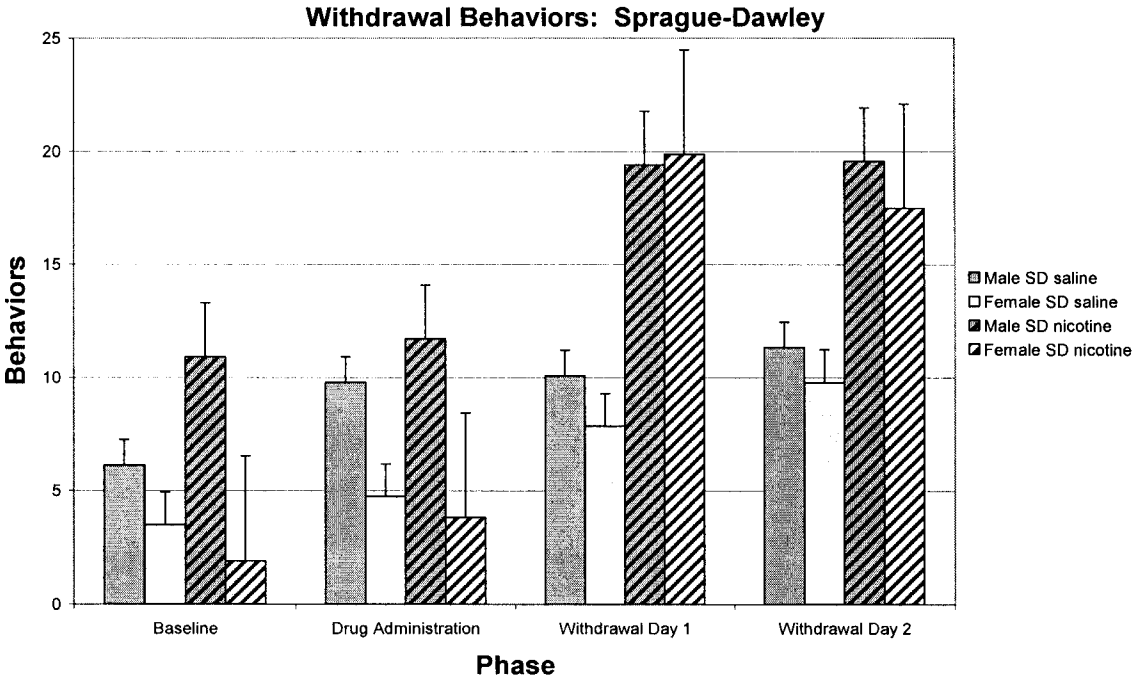

FIGURE 4

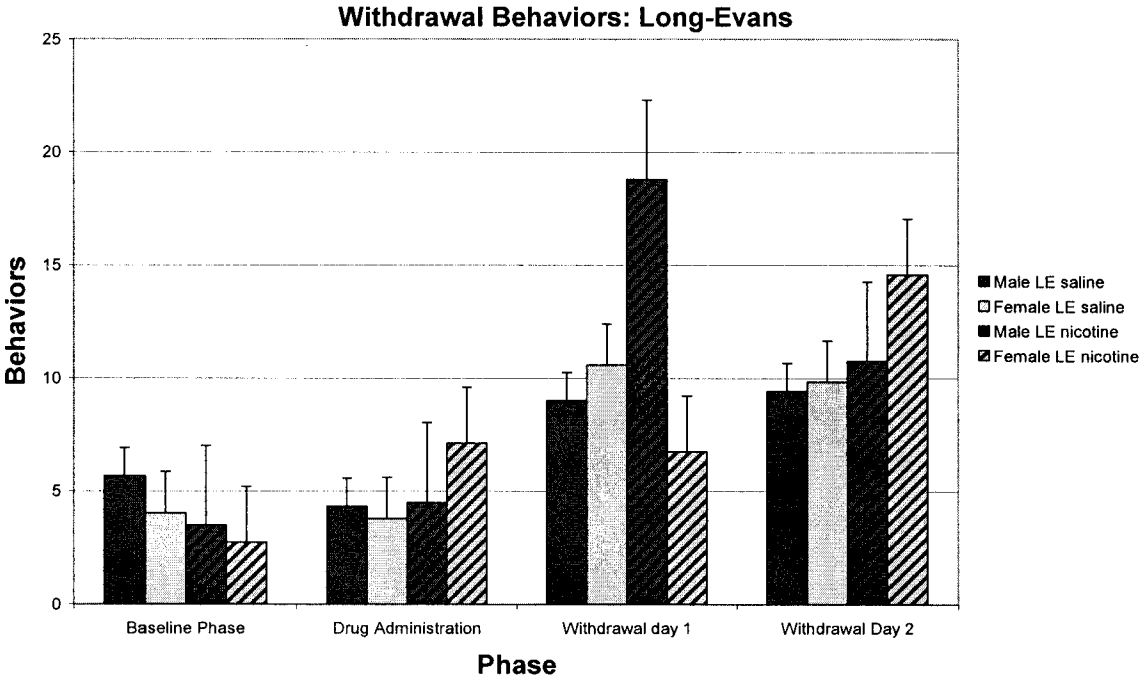

FIGURE 5

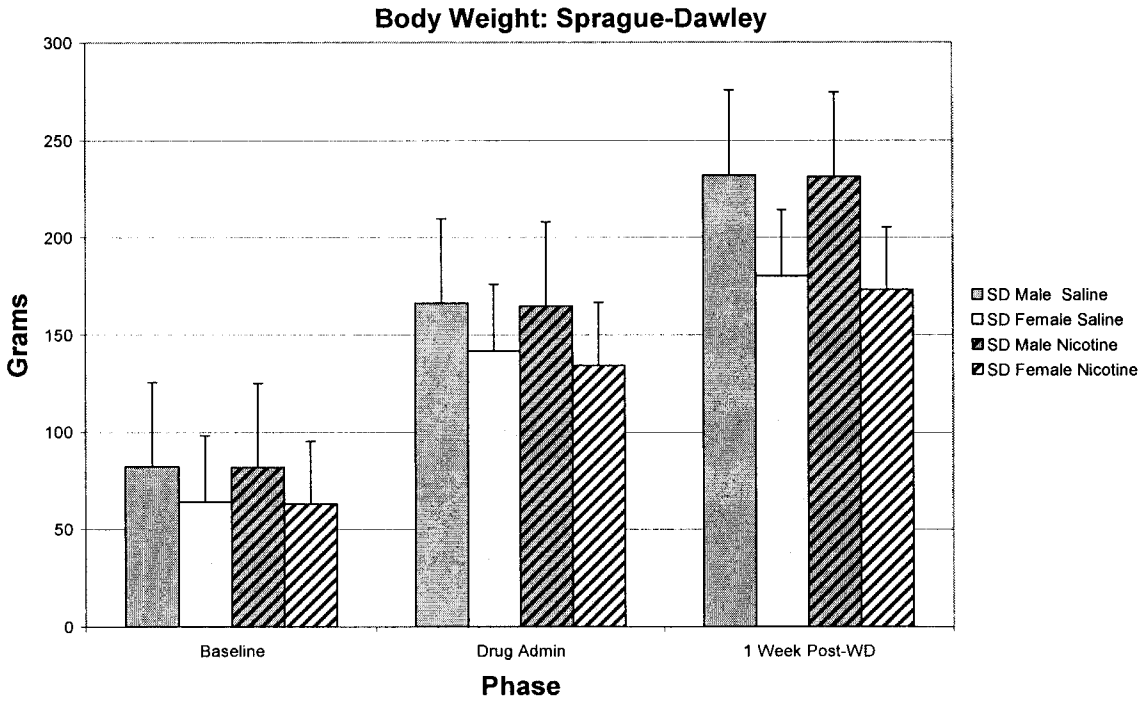

FIGURE 6

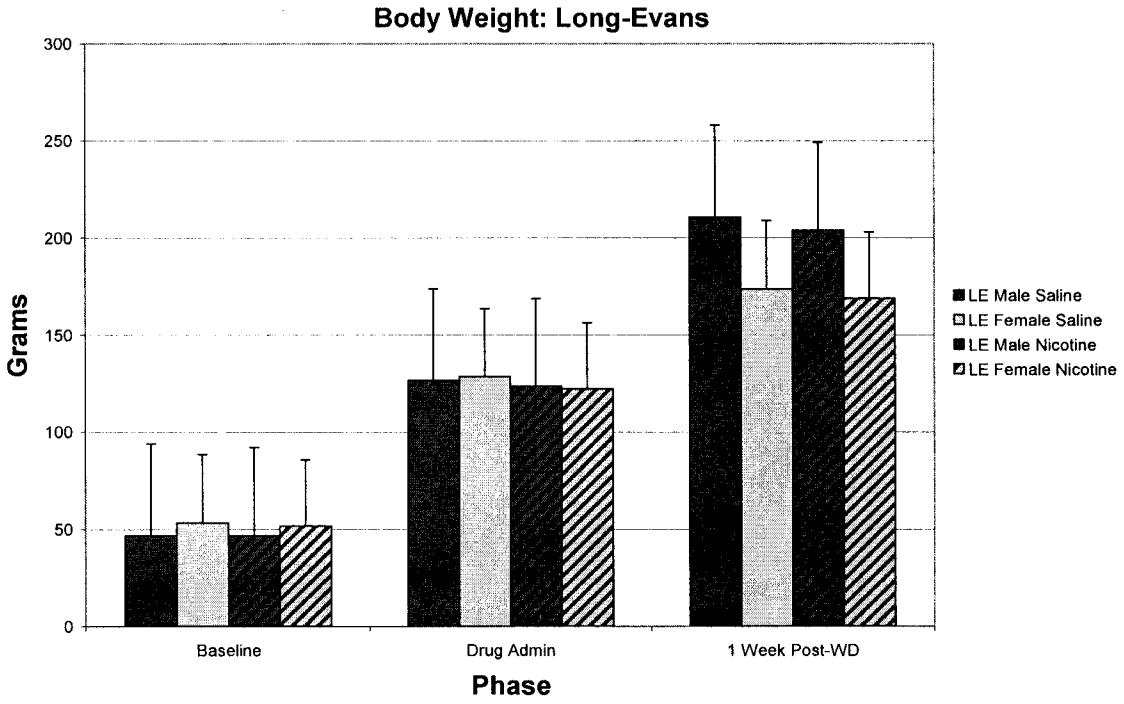

FIGURE 7

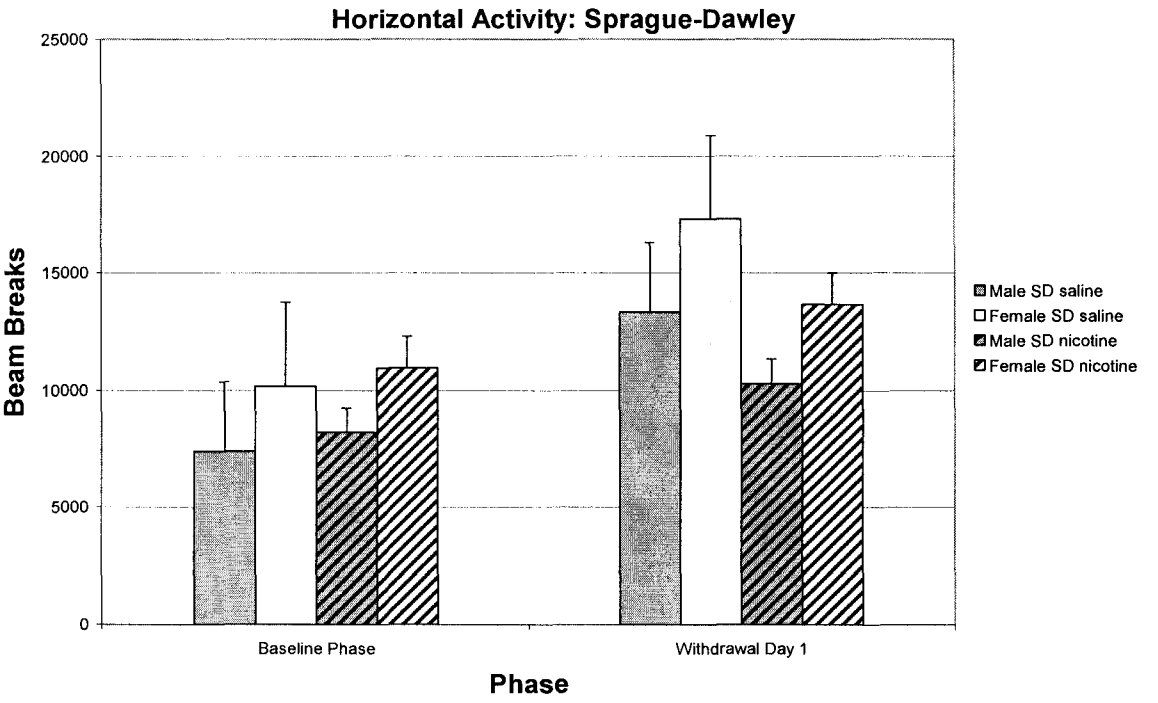

FIGURE 8

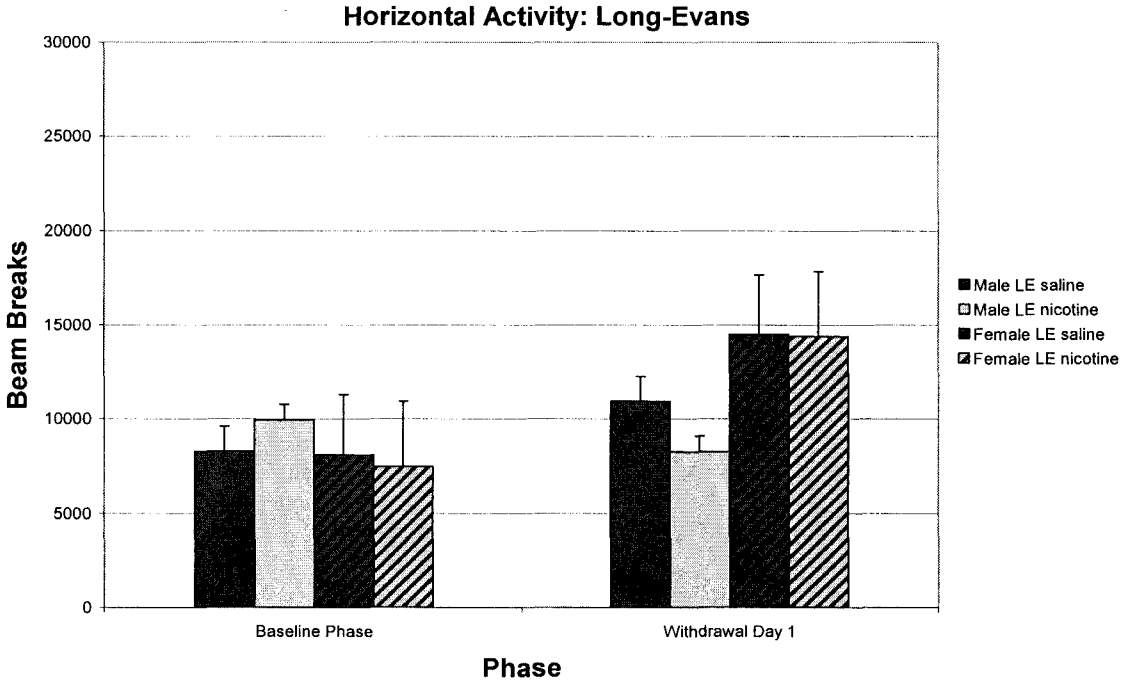

FIGURE 9

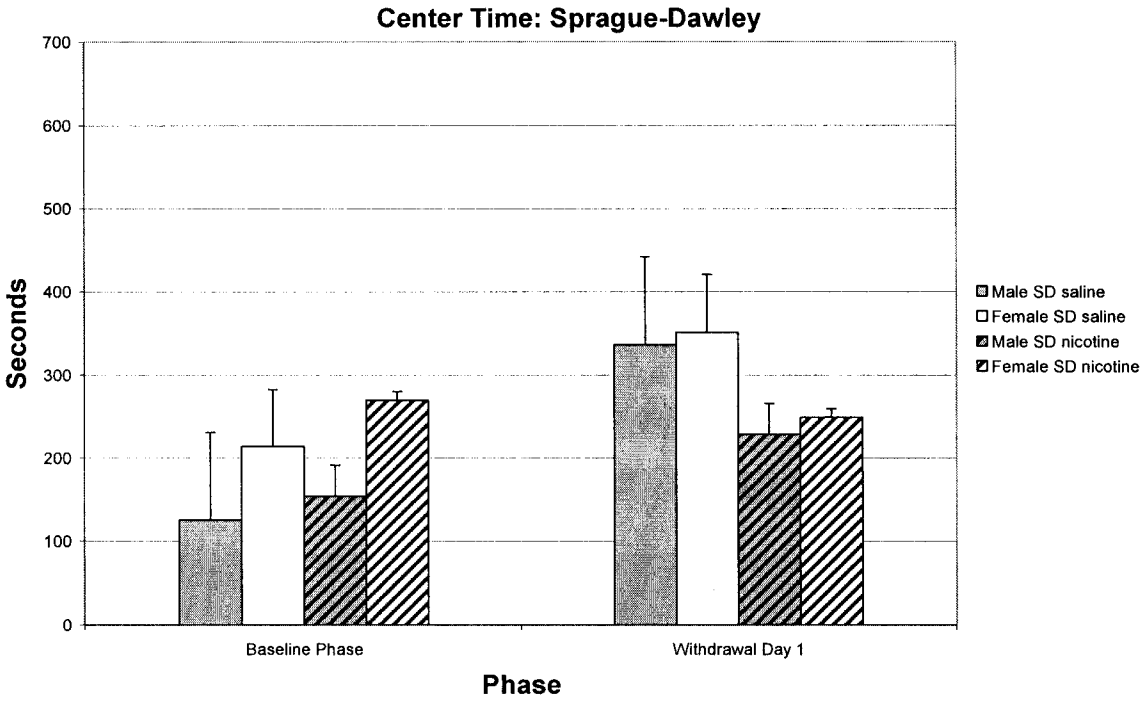

FIGURE 10

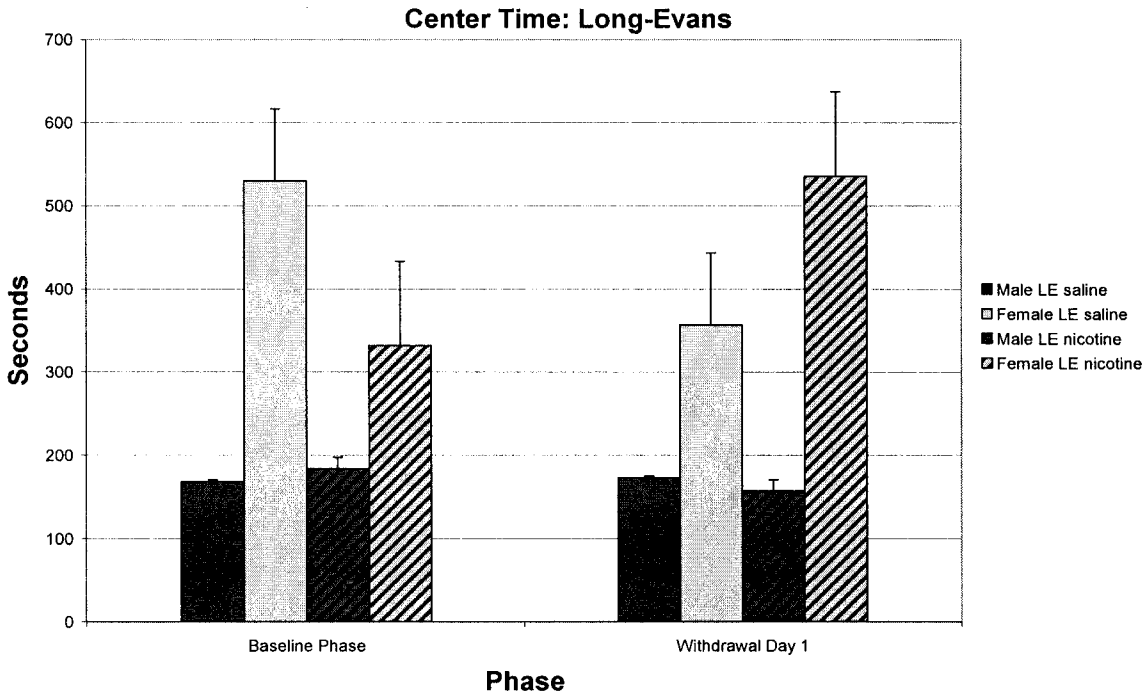

FIGURE 11

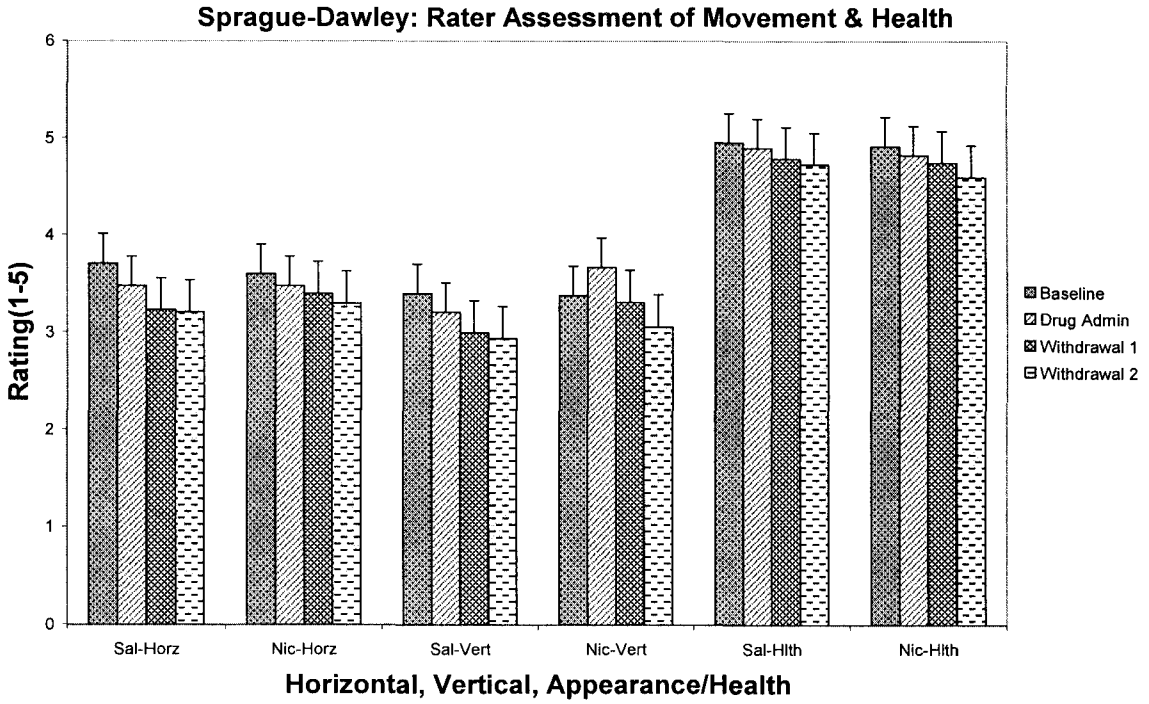

FIGURE 12

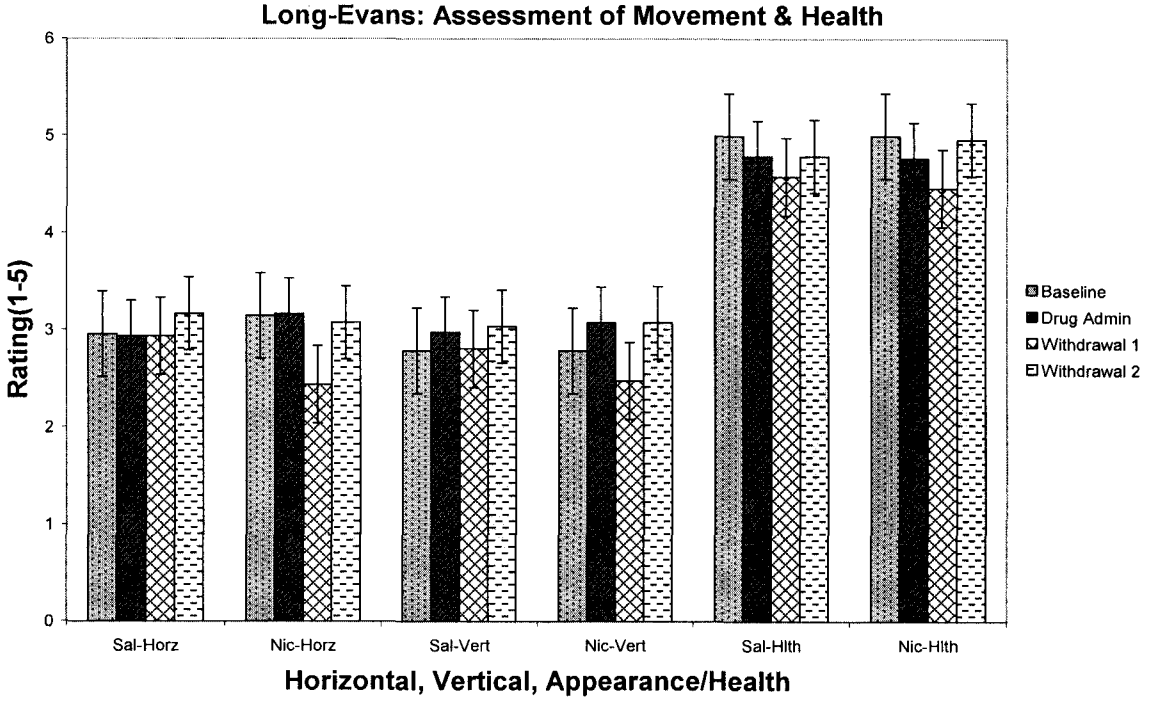

FIGURE 13

Withdrawal Symptoms Observation Data Sheet

Animal Number: _____

Date: _____ Time: _____ Observer Initials: _____

Phase (circle): Baseline Nicotine Withdrawal

Behavior	Number observed	Total
Wet-dog shakes		
Diarrhea		
Mouthing/teeth chattering		
Ptosis		
Abnormal grooming		
Abnormal posture/mvmnt		
Eye blinks		
Other		
	Combined total	

Low High

Horizontal Activity: 1-----2-----3-----4-----5

Vertical Activity: 1-----2-----3-----4-----5

Health: 1-----2-----3-----4-----5